Altered gene expression associated with epizootic shell disease in the American lobster, *Homarus americanus*

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Abstract

Epizootic shell disease is a poorly understood condition that has significantly affected the American lobster fishery in New England (northeastern US) since the 1990s. Here we present the results of a study to identify changes in gene expression in lobsters exhibiting symptoms of epizootic shell disease. Suppressive subtractive hybridization (SSH) was used to compare gene expression between cDNA pools from diseased (symptomatic) and apparently healthy (asymptomatic) lobsters. Subsequently, quantitative real-time polymerase chain reaction (qPCR) was used to measure expression of nine genes that were differentially-expressed in the SSH analysis, in seven tissues (muscle, gill, heart, hepatopancreas, brain, branchiostegite, gonad) dissected from individual symptomatic and asymptomatic lobsters. Expression of arginine kinase (involved in cellular energetics) was significantly decreased in muscle of symptomatic lobsters. Expression of hemocyanin (a respiratory hemolymph protein involved in oxygen transport) was highest in hepatopancreas and showed highly variable expression with a trend toward higher expression in asymptomatic individuals. α2-Macroglobulin (involved in the innate immune system) was most highly expressed in the ovary, particularly of symptomatic lobsters. The ESTs produced through this study add to the fledgling field of crustacean genomics and revealed three genes that could be further evaluated in lobsters of varying shell disease severity, molt stage, and reproductive condition, for possible implication in epizootic shell disease.

Keywords

arginine kinase, biomarkers, crustacean, decapods, hemocyanin, suppressive subtractive hybridization

Abbreviations

SSH, suppressive subtractive hybridization; qPCR, quantitative real-time polymerase chain reaction; AK, arginine kinase; GPCR, G-protein coupled receptor; HACD, hydroxyacyl dehydrogenases; HC, hemocyanin; KCP2, keratinocyte associated protein 2; MBP, mannose-binding protein; A2M, alpha-2 macroglobulin; MVK, mevalonate kinase.

1. Introduction

The American lobster (*Homarus americanus*) is an iconic representative of the Atlantic coast of the United States and Canada and supports a highly valued fishery. Natural lobster populations have undergone dramatic swings in local and regional abundance over the last century, likely due to the combined effects of changes in the physical environment and biological interactions [1]. In southern New England, widespread occurrence of a virulent form of 'shell disease' has been associated with reduced harvests of coastal lobsters beginning in the late 1990s [1, 2].

Historically, several forms of crustacean 'shell disease' have been identified that collectively are characterized by progressive erosion of the exoskeleton from the activity of chitinolytic microorganisms resulting in necrotic lesions, pits, and/or discolorations [3, 4]. Shell disease was first described 80 years ago in lobsters held in impoundments at high densities [5], and was later classified as "impoundment shell disease" by Smolowitz *et al*. [3]. In wild lobster populations, shell disease was documented as early as 1981 [6], and was probably endemic before that time. Endemic shell disease in lobsters received increased attention in response to a 1983 survey that reported a mean prevalence of 12% along the Massachusetts coast [7]. In the 1990s, a much more aggressive form of shell disease emerged, which was characterized by rapid infection and extensive, melanized, and deep lesions [8]. By 2000, Smolowitz and colleagues concluded that the geographic extent and prevalence of the disease qualified it as epizootic [8, 9]. The recent prevalence and severity of epizootic shell disease are unprecedented in the historical record [2, 10].

The factors that have enabled the development and spread of epizootic shell disease have not been characterized. While gram-negative bacteria dominate the microbial community associated with lesions from epizootic shell disease [11], bacterial isolates associated with disease lesions appear to be widely distributed environmental strains that can also be found in association with asymptomatic lobsters. In addition, the disease is not highly contagious, has proven difficult to induce in the laboratory, and has historically occurred at low levels (<2%). Several researchers have suggested that the etiology of epizootic shell disease may involve a combination of environmental stressors leading to increased susceptibility of lobsters to microbial infection [10, 12, 13]. For example, anomalously warm water temperatures have been correlated with spatiotemporal variation in the incidence of epizootic shell disease [6]. Others have examined the role of diet [12, 14, 15], environmental chemicals [16-18] and hypoxia [19].

Our overall working hypothesis is that environmental stressors disrupt lobster physiology, and increase their susceptibility to epizootic shell disease, and that this would be evident in altered gene expression. While comparison of gene expression between symptomatic and asymptomatic lobsters cannot identify direct causality, we hypothesize that it will provide insight into physiological pathways that are associated with epizootic shell disease. To evaluate changes in gene expression, we used suppressive subtractive hybridization (SSH) to identify genes that are differentially regulated in healthy (asymptomatic) *versus* shell-diseased (symptomatic) lobsters. In addition, we used quantitative real-time polymerase chain reaction (qPCR) to quantify the expression of genes identified through SSH. Through this approach, we provide the first description of gene expression patterns associated with epizootic shell disease in lobsters.

2. Materials and methods

2.1 Animals and RNA extractions: Since epizootic shell disease can only be identified through the appearance of characteristic lesions [8], we hereafter refer to diseased lobsters as "symptomatic" and apparently healthy animals as "asymptomatic." One asymptomatic female lobster and one symptomatic female lobster were collected from Cape Cod Bay, Massachusetts in August 2006. Four symptomatic and four asymptomatic male lobsters and four symptomatic and four asymptomatic female lobsters were collected from Rhode Island Sound in May and June 2007. All lobsters were collected using bottom traps. Additional details regarding lobsters used in these two experiments are provided in Table 1. Lobsters were sedated by chilling on ice and sacrificed; tissues were dissected, immediately flash-frozen and stored at -80^{o}C until analysis.

Total RNA was extracted individually from hepatopancreas, brain, heart, gonad, gill, muscle (both tail and claw muscle), and branchiostegite using the Aurum Total RNA Fatty and Fibrous Kit (Bio-Rad) with on-column DNase digestion. RNA yield and purity were quantified using a Nanodrop ND-1000 spectrophotometer and denaturing agarose gel electrophoresis. Aliquots of the RNA were used to create subtractive libraries, and the remainder was retained for qPCR analysis, as described below.

2.2 Suppressive subtractive hybridization (SSH) : Two pairs of hybridizations were conducted; in each pair the asymptomatic and symptomatic lobsters each served as tester and driver. The first hybridization study was conducted using the two lobsters collected from Cape Cod Bay. Equal amounts of RNA were pooled from each tissue, creating a single pool of RNA from the asymptomatic lobster and a second pool of RNA from the symptomatic lobster. Double-stranded complementary DNA (cDNA) was synthesized from 1 μg of each of these two RNA samples using the Super SMART PCR cDNA Synthesis Kit (Clontech), according to the manufacturer's instructions. Suppressive subtractive hybridization was conducted in both directions using the PCR-select cDNA subtraction kit (Clontech) as described previously [20]. Clones were ligated into pGEM-T easy (Promega) and sequenced.

A second subtractive hybridization study was conducted using sixteen lobsters collected from Rhode Island Sound. Two RNA pools were constructed from eight asymptomatic and eight symptomatic lobsters, each containing equal amounts of total RNA from hepatopancreas, heart, gonad, gill, muscle and branchiostegite. Two subtractive libraries were constructed and screened as previously.

2.3 Sequence Analysis: Sequences were compiled, trimmed and clustered using Sequencher Version 4.5 (Gene Codes Corporation) and compared with the NCBI database using the tblastx algorithm. Because the SSH library contained two distinct hemocyanin sequences, we examined the phylogenetic relationships between these and other crustacean hemocyanin sequences. Crustacean hemocyanin sequences were obtained from GenBank and aligned with the ESTs using ClustalW, as implemented in BioEdit [21]. An unrooted phylogenetic tree was constructed using parsimony criteria in PAUP* 4.0 [22].

2.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR): Nine genes of interest were selected from the SSH libraries based on their abundance and known or suspected involvement in lobster immune, energetic, or endocrine function (Table 3). Primers were designed to amplify 75-150 bp fragments of these genes, and 16S rRNA as a housekeeping gene (Table 4). To determine the tissue expression patterns of each gene, cDNA was synthesized from pooled RNA from asymptomatic lobsters and from pooled RNA from symptomatic lobsters for each tissue. cDNA was synthesized from these tissue RNA pools (0.2-2 μg of total RNA per 20 μl reaction, RNA starting quantity varied by tissue) using Omniscript reverse transcriptase (Qiagen) with random hexamers, according to the manufacturer's protocol. Based on this initial tissue profiling, a subset of the nine genes was measured in individual samples of the tissue showing highest expression. RNA from individual ovary, muscle or hepatopancreas

(1 μg per 20 μl reaction) samples was used to make cDNA with the Iscript kit (Bio-Rad). Expression was compared between individual tissues from symptomatic and asymptomatic animals using a student's t-test, with Welch's correction for unequal variance when necessary (Graphpad Prism Software).

All qPCR reactions were performed using the iQ SYBR Green Supermix and a MyCycler iQ Real-Time PCR detection system (Bio-Rad). All samples were run in duplicate wells, and expression was quantified in comparison with a serially diluted plasmid standard, normalized to the expression of 16S rRNA, as described previously [23].

3. Results

Forward and reverse-subtracted libraries were constructed from two independent sets of lobster tissues; thus a total of four libraries were screened. We selected 576 clones for single-pass sequencing, which resulted in 497 sequences longer than 140 bp, after trimming of adapter and vector sequences (Table 2). Of the 497 sequences, 98 were ribosomal, and the remaining 399 sequences have been deposited in the NCBI trace archive (GenBank ID: GO271212-GO271610; dbest ID: 64522502-64522900).

The 399 putative mRNA sequences were compared with sequences in the NCBI database using the blastx algorithm, and the most significant match was recorded with a threshold e-value of 1e-5. The majority (260) of the sequences could not be identified. That is, the sequences were highly similar only to other ESTs or to predicted proteins with unknown function. When the ESTs were clustered, several groups of highly similar or identical sequences became apparent. Most notably, a short (~189 bp) sequence was highly represented in both our symptomatic and asymptomatic libraries (40 and 14 clones, respectively; Unidentified Contig 1 in Table 2). This sequence has previously been identified in lobster tissues (*i.e.*, GO271212 in the present study was identical to DV774281 identified by Stepanyan et al. [24]). Because this sequence does not include a long open reading frame, it may represent 3'-UTR of a highly-expressed gene, a retained intron, or genomic contamination [24].

In some cases, our ESTs were highly similar to sequences identified in other diseased crustaceans. For example, an EST from our asymptomatic-enriched library (GO271347) was highly similar (210/270 identical nucleotides, blastn e =1e-48) to a sequence identified in a SSH library constructed from shrimp injected with the white spot syndrome virus (CX535856, [25]). The function and phylogenetic history of these genes are not known.

Among the genes we have provisionally identified, some of the more abundant transcripts (cytochrome b and c, skeletal muscle actin, myosin) correspond to genes that are typically highly expressed. Like the ribosomal genes, these may represent genes not fully suppressed during the creation of our subtractive libraries (*i.e.*, false positives), and they were not studied further. From the remaining genes, nine were selected for additional screening by qPCR (Table 3); priority was given to genes with a known or suspected involvement in immune function, hormonal signaling or energetic metabolism. The genes were named based on their similarity to annotated genes in the NCBI database: (1) AK (arginine kinase) helps to regulate cellular ATP levels, and changes in expression have been associated with viral infection in other crustaceans [26, 27]. (2) CRUSTINs are antimicrobial peptides with affinity for gram positive bacteria [28]. (3) GPCR (G-protein coupled receptor) is most similar to uncharacterized predicted GPCRs from other arthropods but is moderately similar (blastx e-values 1e-5 – 1e-7) to receptors for cardioaccelaratory peptides, gonadotropin releasing hormone and vasopressin from insects and other taxa. These receptors modulate diverse endocrine functions, including a role for cardioaccelatory peptides in ecdysis [29]. (4) HACDs (hydroxyacyl dehydrogenases) are important for energetic homeostasis, catalyzing a step in the beta oxidation of fatty acids [30]. (5) HC (hemocyanin) is

a respiratory pigment that forms a major component of arthropod hemolymph; some studies have reported changes in expression in response to viral infection [e.g., 31]. (6) KCP2 (keratinocyte associated protein 2) is a protein of unknown function that is upregulated following infection of shrimp with white spot syndrome virus [32]. (7) MBPs (mannose-binding proteins) and (8) A2Ms (alpha-2 macroglobulins) are components of the innate immune response. MBPs bind to mannose molecules on the surface of pathogens [33], while A2Ms bind to and facilitate the clearance of active proteases from bodily fluids [34]. (9) MVK (mevalonate kinase) catalyzes the phosphorylation of mevalonic acid during isoprenoid synthesis, enabling synthesis of the juvenile hormone methyl farnesoate [35].

Arginine kinase was primarily detected in muscle tissue (Figure 1A), and expression was significantly reduced in muscle from symptomatic individuals ($p = 0.0278$, Figure 1B). The hemocyanin transcript was most highly expressed in hepatopancreas (Figure 1C). The mean expression was lower in symptomatic hepatopancreas, but this difference was not statistically significant ($p = 0.126$, Figure 1D). The SSH libraries contained two different hemocyanin sequences (GO271543 and GO271600). Because many forms of hemocyanin have been described and vary in their expression following microbial challenge, we compared our sequences with previously described sequences through a parsimony-based phylogenetic analysis. The hemocyanin transcript measured by qPCR (GO271600) was most similar to previously reported shrimp and lobster hemocyanin sequences (Figure 2). The second sequence was shorter (76 vs. 137 predicted amino acid residues, 42% identity) and grouped in a relatively weakly supported node with a crab hemocyanin sequence (63% bootstrap support). A previous study [31] identified two forms of hemocyanin in the shrimp *Marsupenaeus japonicas* (also called *Penaeus japonicas*), one of which was induced by viral infection ("L" subunit) and one of which remained unchanged ("Y" subunit). Our lobster hemocyanin sequences were equally related to these two shrimp genes; thus the inducibility of crustacean hemocyanin isoforms appears to be species- and gene-specific.

The remaining seven genes (CRUSTIN, GPCR, HACD, KCP2, MBP, A2M, MVK) each were expressed most highly in ovarian tissue (A2M shown in Figure 1E, other genes not shown). Three of these genes (MBP, MVK and A2M) were measured in individual ovarian tissues, but only A2M showed a trend with disease state, with higher mean expression in ovary of symptomatic lobsters ($p=0.0589$, Figure 1F). This difference was nearly statistically significant; statistical power was relatively low because the analysis was restricted to the female animals.

4. Discussion

Using SSH, we generated libraries enriched for genes that vary between symptomatic and asymptomatic lobsters. These libraries were produced from a mixture of lobster tissues and were dominated by abundant transcripts. The only crustacean genome that has been sequenced to date is from the distantly related cladoceran, *Daphnia pulex* [36, 37]. Thus, it is not surprising that over half of the sequences could not be identified, or could only be matched to other ESTs of unknown function. Additional crustacean genomic resources are sorely needed, and the present study and other EST projects [24, 38, 39] will facilitate future molecular studies in crustaceans. In spite of limited genomic resources, several dozen genes could be provisionally identified based on their similarity to annotated sequences. Nine genes of interest were selected for additional characterization and three of these appear to be associated with disease state.

We consistently observed decreased expression of arginine kinase (AK) in the muscle of symptomatic lobsters. AK is a highly conserved phosphotransferase that helps to regenerate adenosine triphosphate (ATP) and plays an important role in cellular energetics. The AK sequence has previously been described

in lobsters and other crustaceans [40], and increases in AK activity in lobster hemolymph, which indicate enzyme leakage across muscle cellular membranes, have been proposed as a useful indicator of systemic disease state [41]. In some euryhaline crabs, AK activity (but not RNA expression) is induced in the gills by osmotic stress [42]. Increased AK protein levels were reported in gills of *Penaeus vannamei* infected with yellow head virus [26]. Wang et al. [27] reported decreased expression of AK in cephalothorax of shrimp infected with white spot syndrome virus. Our results suggest that infection with shell disease may create an energetic drain that physiologically compromises muscle function. Future assays of AK activity in both muscle tissues and hemolymph of epizootic shell diseased lobsters would be informative in this regard.

We identified two different hemocyanin (HC) sequences in our SSH library and observed highest HC expression in hepatopancreas. Crustaceans typically have several forms of HC, which function primarily as oxygen carriers [43] but can also play a role in wound healing and innate immunity. Some forms of HC can be cleaved to produce antimicrobial peptides [44, 45] or to acquire phenoloxygenase activity [46, 47]. The form of HC measured in our study is most similar to a crayfish protein that has been shown to produce antimicrobial peptides [45]; thus, it seems possible that the lobster protein is similarly cleaved to produce antimicrobial peptides. HC mRNA expression in lobster hepatopancreas observed in our study is consistent with previous characterization of hepatopancreas as the primary site of crustacean HC synthesis [48] and mRNA expression [49]. Some crustacean HCs are upregulated in pathogen-resistant animals or induced by microbial challenge, while others are not [31, 49]. Phylogenetic analysis did not allow us to match our sequences with sequences that are known to be induced by microbial challenge in other crustaceans. While mean HC expression was lower in symptomatic lobsters, expression was highly variable and not statistically different from expression in asymptomatic lobsters. An additional study with a larger sample size is warranted.

α2-Macroglobulins (A2Ms) are highly abundant proteins both in vertebrate plasma and arthropod hemolymph. While the best-characterized function of A2Ms is the binding and clearance of destructive endogenous and exogenous proteases, A2Ms are relatively unreactive with essential endogenous proteases. For instance, A2M found in the plasma of crayfish shows only weak inhibitory activity against the protease responsible for activating the prophenoloxidase defense system, which is also present in the plasma and responsible for melanin formation during pathogen invasion [34, 50, 51]. In our study, an A2M-like gene was expressed primarily in ovary with a trend toward increased expression in symptomatic lobsters. Although the difference was not significant, the statistical power was relatively low (due to the small sample size for ovarian tissue). Most studies have not explicitly quantified A2M expression in crustacean ovary, although one study reports weak expression in ovary of kuruma shrimp [52]. A2M expression has been more thoroughly studied in mammalian ovaries, where it regulates protease activity necessary for ovulation and associated tissue remodeling [53, 54]. Other studies of A2M expression in crustacean tissues have found that A2M transcripts are expressed most strongly in hemocytes [52, 55, 56] and that expression can be induced by microbial challenge [52, 55, 57]. Hemocytes were not analyzed in our study, and we anticipate that we might find elevated expression of A2M in hemocytes of symptomatic lobsters. A2M expression in relation to lobster epizootic shell disease merits additional study, including quantification in hemocytes and a larger number of ovarian samples.

Our study is the first to apply SSH and qPCR to increase our understanding of the potential causes for the recent outbreak of epizootic shell disease in southern New England lobsters. While the current study does not unequivocally point to causes or mechanisms of lobster shell disease initiation or progression, it identifies three genes (AK, HC and A2M) that appear to be associated with epizootic shell disease and contributes to the fledgling of crustacean genomics. Future work should focus on evaluating

lobsters of varying disease severity, molt stage, and reproductive condition. Notably the symptomatic lobsters used in this study were all moderately to severely diseased. Additional studies are particularly needed to identify markers associated with early disease incidence and susceptibility.

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Tables

Table 1. General characteristics of lobsters used in molecular analyses.

SSH 1

Gender	Disease condition	Carapace Length	Weight
Female	50% lesions	12 cm	697 g
Female	Asymptomatic	11.4 cm	541 g

SSH 2 and qPCR studies

Gender	Disease condition	Carapace Length	Weight
Male	50% lesions	9.6 cm	600 g
Male	50-100% lesions	7.8 cm	365 g
Male	> 75% lesions	7.9 cm	436 g
Male	> 75% lesions	8.8 cm	578 g
Female	> 75% lesions	8.2 cm	485 g
Female	75% lesions	7.4 cm	283 g
Female	> 75% lesions	7.3 cm	266 g
Female	25-50% lesions	7.9 cm	365 g
Male	Asymptomatic	5.3 cm	107 g
Male	Asymptomatic	7.4 cm	308 g
Male	Asymptomatic	8.0 cm	401 g
Male	Asymptomatic	8.5 cm	449 g
Female	Asymptomatic	7.2 cm	336 g
Female	Asymptomatic	6.8 cm	248 g
Female	Asymptomatic	6.7 cm	197 g
Female	Asymptomatic	6.8 cm	249 g

Table 2. General characteristics of lobster ESTs (Expressed Sequence Tags) from suppressive subtractive hybridization. Some categories represent multiple distinct transcripts: subunits of cytochrome b and c have been grouped, and "myosin" and "actin" each refer to multiple isoforms or gene regions. C1-C22 refer to unidentified ESTs clustered into highly similar "contigs." For each gene or contig, one representative Genbank ID is listed.

	GenBank ID	Upregulated in Symptomatic Lobsters		Downregulated in Symptomatic Lobsters	
Study Gene Annotation		SSH1 (GO271212-GO271284, GO271605)	SSH2 (GO271350-GO271477, GO271607, GO271610)	SSH1 (GO271285-GO271349, GO271606)	SSH2 (GO271478-GO271604, GO271608-GO271609)
Total		82	163	84	168
Ribosomal RNAs		8	33	18	39
Putative mRNAs		74	130	66	129
Unidentified		44	87	43	86
C1	GO271212	29	11	7	7
C2	GO271375		2		
C3	GO271214	5	2	3	
C4	GO271369		2		
C5	GO271497				2
C6	GO271221	2			
C7	GO271327		3	1	2
C8	GO271364		2		
C9	GO271415		2		
C10	GO271434	2		2	3
C11	GO271455		2		1
C12	GO271451		3		3
C13	GO271263	1	1		
C14	GO271347			3	1
C15	GO271312		1	4	2
C16	GO271558				2
C17	GO271527		1		2
C18	GO271404		2		3

C19	GO271550		1		1
C20	GO271394		3		
C21	GO271512		1		1
C22	GO271337		1	1	
Other		5	47	22	56
Identified mRNAs		30	43	23	43
Arginine kinase	GO271571		1		3
Cysteine proteinase	GO271459		2		2
Cytochrome b or c	GO271434		8		3
Hemocyanin	GO271600				2
Keratinocyte Associated	GO271423		2		
Muscle LIM protein	GO271411				3
Myosin	GO271255	16		7	1
Ornithine decarboxylase antizyme	GO271403		2		
Reproductive-related	GO271389		2		
Skeletal muscle actin	GO271225	11			2
Mannose binding protein	GO271257	1	2	3	1
Crustin	GO271426		1	2	2
Xbox-binding protein	GO271285			2	
Other (single ESTs)		2	23	4	24

Table 3. ESTs chosen for characterization by qPCR. Reference species indicates the organism and accession number from the most significant match found by the blastx algorithm. The library category indicates whether the gene was predicted to be upregulated in asymptomatic or symptomatic animals. The number of ESTs in the library is also indicated. In two cases (AK and MBP) the gene was found in both the symptomatic and asymptomatic libraries. The E-value indicates the probability that another alignment would have a higher degree of similarity due to chance.

GenBank ID	Abbreviation (gene name)	Reference species (Accession Number)	Library (# of ESTs)	length bp	E-value
GO271571	AK (Arginine kinase)	*Homarus gammarus* (P14208)	Asymptomatic (4); Symptomatic (1)	485	7e-76
GO271287	CRUST (crustin-like protein precursor)	*Homarus americanus* (ABM92333)	Asymptomatic (2);	492	6e-40
GO271592	GPCR (G-protein coupled receptor)	*Ixodes scapularis* (EEC06829)	Asymptomatic (1)	673	1e-13
GO271496	HACD (hydroxyacyl dehydrogenase)	*Aedes aegypti* (XP_001659937)	Asymptomatic (1)	225	6e-13
GO271600	HC (Hemocyanin)	*Pacifastacus leniusculus* (AF522504)	Asymptomatic (2);	516	6e-76
GO271423	KCP2 (Keratinocyte associated protein 2)	*Litopenaeus vannamei* (ABI93175)	Symptomatic (2)	144	8e-16
GO271317	MBP (mannose-binding protein)	*Pacifastacus leniusculus* (AAX55747)	Asymptomatic (2); Symptomatic (5)	334	9e-18
GO271388	A2M (alpha-2-macroglobulin-like)	*Macrobrachium rosenbergii* (ABK60046)	Symptomatic (1)	217	3e-4[1]
GO271349	MVK (mevalonate kinase-like)	*Danio rerio* (CAM15186)	Asymptomatic (1)	472	6e-17

[1] This e-value is above the cutoff we used to annotate other genes; however, the relatively low similarity is most likely because the lobster EST is homologous to a poorly conserved part of the A2M gene between the highly conserved thioester motif and receptor binding domain.

Table 4. Sequences of oligonucleotide primers used in qPCR assays. Full gene names are shown in Table 3.

Gene	Primer sequences
AK	F: 5’-CATCGCAAAGTTGGAGGAAGG-3’
	R: 5’-GCCAGTCTTCTTGGCCTTGAG-3’
CRUST	F: 5’-GGTGCAATTTCCCAGAGGATG-3’
	R: 5’-GAACCTTGCGCACGTTATATGC-3’
GPCR	F: 5’-ACCTTCACGAGACGCTGGAAC-3’
	R: 5’-TCCATCGGTGTTCATCTGCTG-3’
HACD	F: 5'-GCCCTGACTTAGCAAGATCC-3'
	R: 5'-AGGCAGGTCACAGATCACAG-3'
HC	F: 5’-ATCAGCGTCGTGGATCAGTTG-3’
	R: 5’-TGCTCGACACCTTCTGGACTG-3’
KCP2	F: 5’-CCGAACATGACGTTTTCCAAG-3’
	R: 5’-GACCCAGTTGGGTGCAACAAG-3’
MBP	F: 5’-CGGGCATACCACTTCTCTTGG-3’
	R: 5’-GCTGACCGCCTGGAAGTTATG-3’
A2M	F: 5’-TCCAGCTGCCCAGTGTGTAAG-3’
	R: 5’-ACTCGGCATGAGGCAACTGAG-3’
MVK	F: 5’-CGGGTCCGAACACATCTCAC-3’
	R: 5’-TCGTGCATGGTTTTCATCGTC-3’
16S	F: 5’-AATACCGCGGCCCTTTAGTTTG-3’
	R: 5’-TTGGTGTGGGTTAAGGAACTCG-3’

Figure Captions

Figure 1: Gene expression in asymptomatic (white bars) and symptomatic (black bars) lobsters as measured by qPCR. (A, C, and E) Expression in pooled cDNAs from tissues used in SSH. Each bar represents a single pooled sample. Due to large differences in expression among tissues, a log scale is shown. Tissue types are muscle, M; gill, G; heart, H; hepatopancreas, HP; brain, B; testes, T; and ovary, O. (B, D, and F) The two bars at the left of each plot indicate mean ± standard error expression for asymptomatic and symptomatic animals, respectively. The remaining bars indicate expression in individual samples used to comprise tissue pools.

Figure 2: Unrooted parsimony-based phylogenetic analysis of selected crustacean hemocyanin amino acid sequences. Taxa used were *Cancer magister, Marsupenaeus japonicas, Litopenaeus vannamei, Pacifastacus leniusculus,* and *Homarus americanus.* Numbers at nodes indicate the percentage of 1000 bootstrap replicates supporting a given clade. GenBank Accession numbers are indicated under each species name. Subunit designations are those reported in GenBank submissions and associated papers. Two hemocyanin ESTs (designated "novel sequence" and indicated in bold) were detected within the subtractive libraries in the present study. Expression of one of these (indicated with asterisks), was measured by qPCR.

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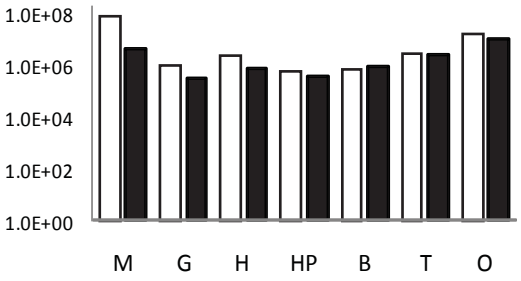
A.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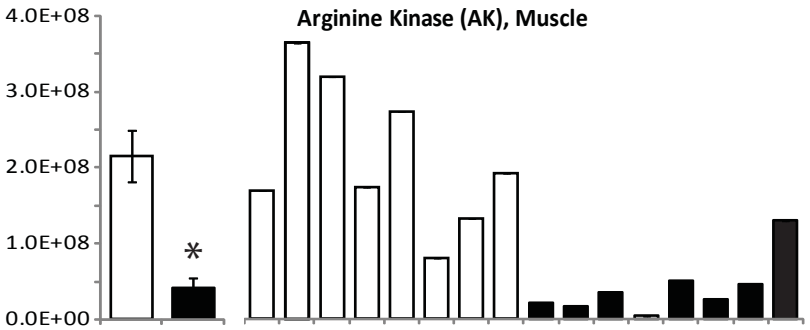
1.0E+04
1.0E+02
1.0E+00
M G H HP B T O

B.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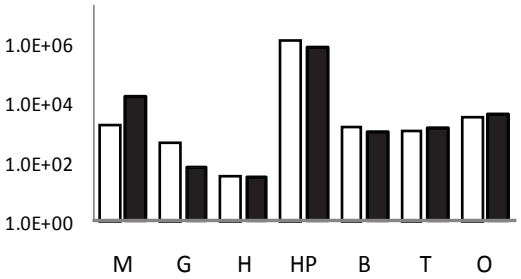
2.0E+08
1.0E+08
0.0E+00
*

C.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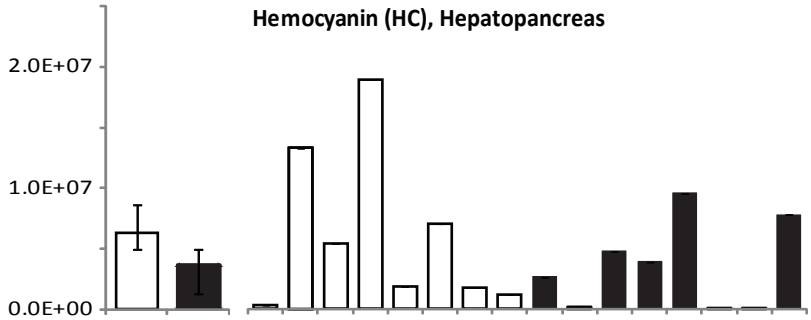
D.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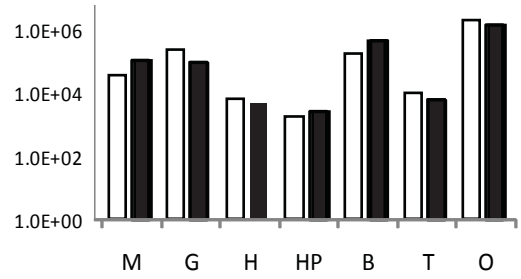
E.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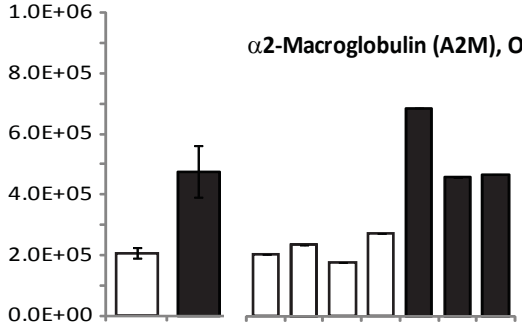
M G H HP B T O

F.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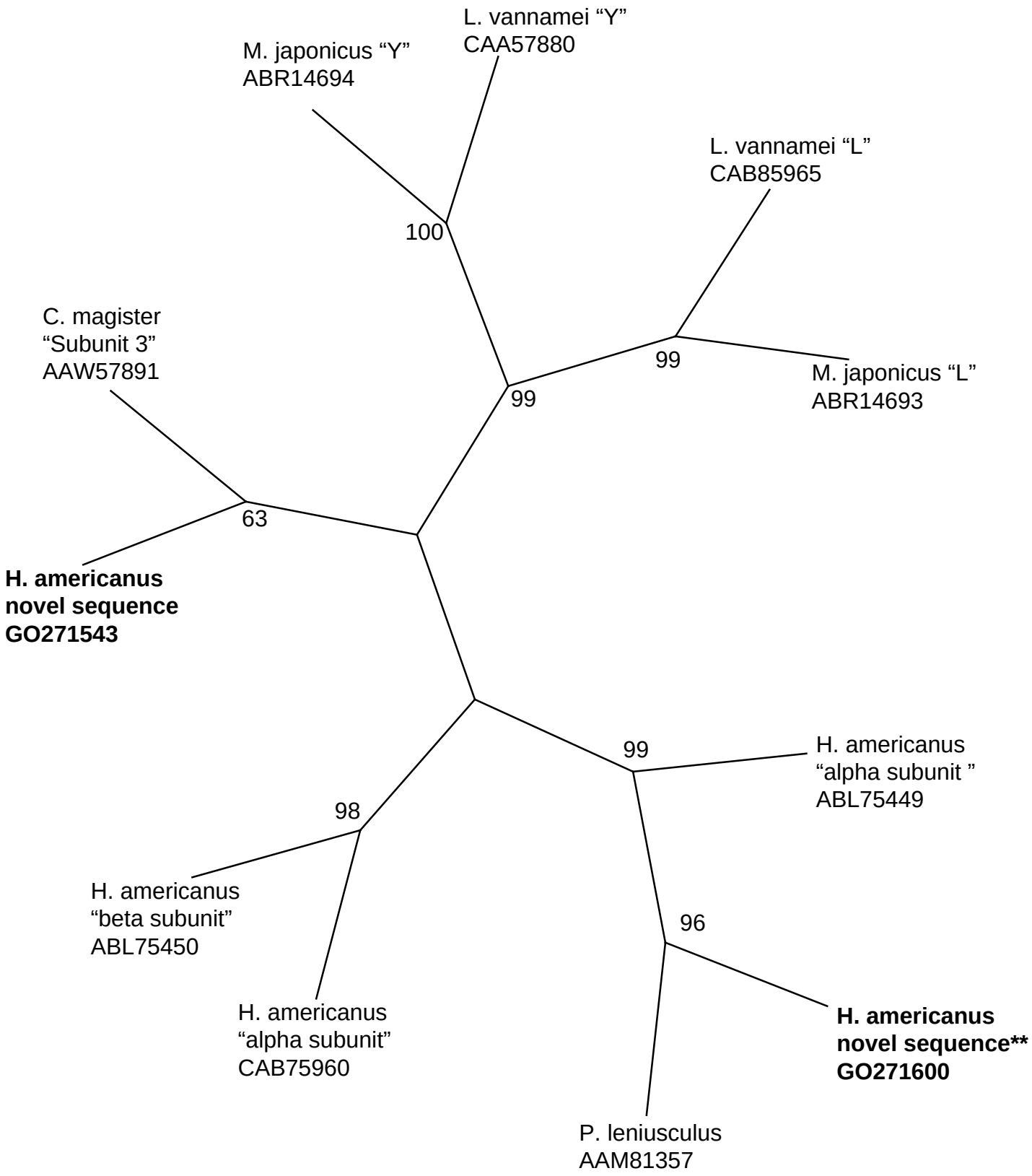
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M. japonicus “L”
ABR14693
99
63
H. americanus
novel sequence
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H. americanus
“alpha subunit ”
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H. americanus
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ABL75450
96
H. americanus
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H. americanus
novel sequence**
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P. leniusculus
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